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14. ABSTRACT
Our data demonstrates that WBP-2 is recruited onto the hormone responsive promoters in the presence of hormone and it specifically enhances the transactivation functions of PR and ER. Our data also demonstrates that WBP-2 contains an intrinsic activation domain and the cPPXY of WBP-2 is essential for its coactivation and intrinsic activation functions. Our preliminary data also demonstrates that the WBP-2 binding protein, YAP1 enhances PR and ER transactivation but YAP1's coactivation function is absolutely dependent on WBP-2. Furthermore, cPPXY motif of WBP-2 and WW-domain of YAP1 is required for YAP1 to work as a transcriptional coactiva-tor. Additionally, our data also indicate that the coactivation functions of WBP-2 and YAP1 are suppressed by WWOX1, suggesting that WWOX1 may regulates the transactivation functions of ER and PR by antagonizing the functions of WBP-2 and YAP1. Taken together our data estab-lished the role of WBP-2 and YAP1 as coactivators and WWOX1 as a repressor for ER and PR transactivation pathways.

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Introduction

Estrogen (ER) and Progesterone (PR) Receptors: Estrogen receptor-α (ER) and progesterone receptor (PR) are members of a superfamily of hormone-regulated transcription factors that stimulate gene expression in response to estrogens and progesterones respectively. These receptors contain common structural motifs, which include a less well-conserved amino-terminal activation function (AF-1) that affects transcription efficiency, a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determines target gene specificity, and a carboxy-terminal hormone-binding domain (HBD). The HBD contains activation function-2 (AF-2), the region that mediates the hormone-dependent activation function of receptors. In order to activate gene transcription, the ER and PR undergo a series of well-defined steps. When bound to hormone, these receptor undergo a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with co-activators and recruitment of chromatin modifying enzyme activities such as histone acetyl transferase activity (HAT), methyl transferase activity, ATPase activity, ubiquitin-conjugation activity and ubiquitin-protein ligase activity, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex [37-40]. These events are followed by up- or down-regulation of target gene expression. However, these receptors may also be converted into active forms even in the absence of hormones in target cells. The mechanism of hormone-independent activation of ER and PR has not been understood fully yet but it may involve the bypassing of any one of the above mentioned steps of hormone-dependent activation [41, 42].

Coactivators of ER and PR: Nuclear hormone receptor coactivators are molecules that interact with activated receptors and stimulate receptor-mediated transcription of target genes. There are now ~200 published nuclear hormone receptor coactivators that work with ~ 48 nuclear receptors [6, 8, 9, 11]. The most widely studied coactivators include members of the p160 family of coactivators; SRC-1 (steroid receptor coactivator-1) [43], SRC-2 (TIF-2; transcription intermediary factor-2/GRIP-1; glucocorticoid receptor interacting protein-1) [44, 45], SRC-3 (p/CIP; p300/CBP interacting protein/ACTR; activator of thyroid and retinoid acid receptors/AIB-1; amplified in breast cancer-1/RAC-3; retinoid acid receptor coactivator-3/TRAM-1; thyroid receptor activator molecule-1) [46-49], the CBP (CREB-binding protein)/p300 family [50], coactivator-associated arginine methyltransferase (CARM-1) [51, 52], and E6-AP [10]. We have previously reported the cloning and characterization of E6-AP as a novel dual function steroid hormone receptor coactivator. Additionally, we also demonstrated that the E2 ubiquitin-conjugating enzyme, UbcH7, acts as a coactivator of steroid hormone receptors [53]. Initially, it was thought that coactivators act as adaptors and provide a bridge between DNA binding transcription factors and the general transcription machinery. This simple scenario of coactivator action turned out to be much more complex. It has been shown that coactivators can mediate chromatin modifications either through acetylating reactions mediated by histone acetyl transferases or through nucleosome remodeling complexes [6, 8, 11]. Coactivators are predicted to have many activities in addition to the initiation of transcription, such as mRNA transport from the nucleus, mRNA translation, and posttranslational modifications of the synthesized protein. That coactivators possess stratified actions in the entire process of transcription-translation reflects the fact that they do not act alone but rather as part of multiprotein complexes. These multisubunit entities, containing many individual enzymatic activities, represent a complex machine that is able

to concentrate and link diverse enzymes, and the processes that they regulate, together in one place [6, 8, 11]. In this way, the coactivator complex executes the coactivator's final agenda—that is, to see a particular gene expressed as a mature functional protein.

Transcription is a highly dynamic and orderly process involving many subreactions (multiple steps of initiation, elongation, splicing, and termination) [54]. Given that so many nuclear hormone receptor coactivators have been identified, there is certainly no shortage of them to participate in the wide variety of transcription subreactions. But why would a cell possess such a cumbersome transcriptional apparatus? The answer may lie in the fact that mammals are substantially more complex than organisms such as yeast, worm, and the fruit fly, which have far fewer nuclear receptor coactivators. For instance, only a single nuclear receptor coactivator (Taiman/dAIB1) has been identified in fruit flies so far [55].

For transcription to proceed, there need to be histone modifications (such as acetylation and methylation), ATPase-dependent chromatin remodeling, initiation of transcription, elongation, alternative RNA splicing and mRNA processing, and termination. The focus of coactivator enzymatic activities in these processes has centered on the posttranslational modification of histones and chromatin [56, 57]. However, it is becoming clear that nuclear hormone receptors and their coactivators are also subject to posttranslational modification [58, 59]. The posttranslational targeting of nuclear hormone receptors and their coactivators is important because these modifications influence the expression of functionally related groups of genes. *Identification of new coactivators will provide the prime source for the discovery of new molecular events in transcriptional reactions.*

As mentioned above that coactivator proteins form multiprotein complexes to efficiently regulate target gene transcription. Recently, we have identified novel protein named WBP-2 as an E6-AP interacting protein and show that it specifically modulates ER and PR functions. WBP-2 was previously shown to interact with human YAP1 via the WW-domain of YAP1 protein [12]. The WW-domain is characterized by 35-40 semi-conserved amino acids, which are involved in protein-protein interaction. WBP-2 interacts with the WW-domain via a short proline-rich motif (PPXY) with the consensus sequence of four consecutive prolines followed by tyrosine [16, 31, 35]. It has been speculated that WBP-2 plays role in transcription, but its exact function in steroid hormone receptor-dependent transcription has not been defined yet. Additionally, it has been suggested that YAP1 may also regulate transcription by acting as a coactivator for several transcription factors but what role YAP1 plays in steroid hormone receptor-mediated gene transcription remains unknown. *The main goal of this proposal is to decipher the mechanism by which WBP-2 and YAP1 regulates ER and PR-mediated transcription.*

WBP-2 as a Coactivator: WBP-2 is a 30 kDa protein that contains three proline-rich motifs known as PPXY motifs. WBP-2 was originally identified as a putative ligand that binds to the WW-domain of YAP1 with relatively high affinity and specificity. The PPXY motifs of WBP-2 are distinct from the PXXP ligand consensus sequence of Src homology domain 3 (SH3) domains [16, 31, 35]. The exact function of the WBP-2 and its PPXY motifs remain unknown. Recently, we cloned WBP-2 as E6-AP interacting protein. Despite the fact that E6-AP does not contain a WW-domain, our data clearly demonstrate that WBP-2 can also interact with proteins that do not contain a WW-domain suggesting a new type of protein-protein interaction between PPXY motif containing protein and *HECT* (homologous to the E6-AP carboxy terminus) domain con-

taining proteins [60, 61]. Additionally, we also found that WBP-2 specifically directly interacts with ER and PR. Our observation that WBP-2 can interact with proteins that do not contain WW-domain is also consistent with a previously published study that suggests WBP-2 interacts with the transcription factor Pax8 [14]. Previously, it has been suggested that WBP-2 act as a transcriptional adaptor for Pax8 [14]. We have demonstrated a role for WBP-2 in steroid hormone receptor functions. We have shown that WBP-2 specifically enhances the hormone-dependent transcriptional activity of ER and PR suggesting that WBP-2 can act as transcriptional coactivator of selected steroid hormone receptors. Furthermore, we have also demonstrated that the most carboxy terminal PPXY motif (cPPXY) of WBP-2 is required for its coactivation function, suggesting a role for the PPXY motif in transcription. Together, our results indicate the role of WBP-2 as a coactivator in modulating ER and PR functions [12]. Previously, it has been postulated that the PPXY motif plays an important role in transcriptional activation since the PPXY motif is present in the transcriptional activation domains of a wide range of transcription factors including c-Jun [62], AP-2 [63], NF-E2 [22], C/EBPá [64] and PEBP2/CBF [30]. Our data indicate a role for the PPXY motif and WBP-2 in ER and PR transactivation, but the exact mechanism by which WBP-2 acts as a coactivator and the role of the PPXY motif in ER and PR-dependent gene activation is not fully understood. Furthermore, *the actual target proteins of the PPXY motif that confer transcription stimulation activity have not yet been identified.* Therefore, there is a clear need *to understand the role of WBP-2 and its PPXY motif in ER and PR-mediated gene activation and transcription.*

WW-Domain Containing Proteins as Modulators of ER and PR Function: Specific protein-protein interactions and multiprotein complexes are important for a multitude of cellular processes including gene transcription. As mentioned above that WBP-2 binds to proteins that contain WW-domain. WW-domains are small protein modules composed of 38-40 amino acids and fold as a stable, triple stranded beta-sheet. The name refers to two signature tryptophan (W) residues that are spaced 20-22 amino acids apart and are present in most of the WW-domains. In some instances, however, the first or the second conserved tryptophan is substituted by other aromatic residues. WW-domains bind to their ligands via the proline rich peptide motifs. WW-domains can be grouped into four classes according to their ligand binding preference. Class I includes WW-domains that bind to the PPXY core sequence which is present in WBP-2. The example of Group I WW-domain containing protein is YAP1. Class II WW-domains prefer ligands containing a stretch of prolines interrupted by a leucine. Class III includes WW-domains interacting with proline-rich sequence that contains arginines or lysines. WW-domains binding phosphoserine or phosphothreonine followed by a proline residue are grouped in class IV. WW-domains are found in both cytoplasmic and nuclear proteins, WW-domains containing proteins are involved in a wide variety of cellular processes, including ubiquitination, nuclear signaling, cell cycle control, transcriptional regulation and the recruitment of signaling proteins [28, 31, 35]. Since, WW-domains bind to the PPXY motif and WBP-2 has been shown to contain PPXY motifs, an important step toward characterizing coactivation function of WBP-2 is to identify to which particular WW-domains it bind, and hence determine the mechanism by which it act as a coactivator and also find out with which signaling pathway it is involved. In order to identify the WW-domains that could interact with the WBP-2 protein, we utilized Panomics' TranSignal WW-domain Array (Panomics Inc., CA, USA). This array contains 67 different human WW-domains from 42 different proteins. Our screening data suggest that WBP-2 interacts with a wide variety

of WW-domain containing proteins (please see preliminary data section) including YAP1, a transcriptional coactivator and WWOX1, a tumor suppressor.

YAP1 was originally identified as a protein binding to the SH3 domain of the Yes proto-oncogene product that belongs to the *src* family of protein-tyrosine kinases. YAP1 is a 65 kDa protein with a well characterized WW-domain [13]. Recently, a splicing variant that encodes human YAP with two WW-domains has been identified. YAP1 with a single WW-domain, known previously as human YAP, is renamed as YAP1 and the new YAP with two WW-domains is named as YAP2. The difference in the amino acid sequence between YAP1 and YAP2 is an insertion of the additional WW-domain in YAP2. YAP1 and 2 contain activation domain which is similar to VP-16 activation domain. Roles of YAP1 and 2 in transcription are not well defined [21]. Recently, YAP1 and YAP2 have been shown to be transcriptional coactivators for several genes but their transcriptional coactivation functions are dependent on PPXY motif containing proteins as in the case of ErbB4 (PPXY motif containing protein) signaling [21]. YAP1 and YAP2 interact with ErbB4 and are recruited to its specific target gene promoter. It has been suggested that YAP2 is a stronger coactivator of transcription than YAP1. Furthermore, phosphorylation of YAP1 and YAP2 by specific kinases regulates their cellular distribution and transcriptional coactivation functions. It is shown that Akt-dependent phosphorylation of YAP1 and YAP2 at Serine 127 induces the interaction of YAP1 with 14-3-3 and promote YAP1 and YAP2 localization to the cytoplasm resulting in loss from the nucleus where they functions as coactivators of transcription [65]. Our preliminary data also indicate a role for YAP1 in steroid hormone receptor-mediated transactivation function. We show that YAP1 expression itself has no significant effect on ER and PR-mediated transcription [12], however, when YAP1 was coexpressed with WBP-2, it could selectively modulate ER and PR-dependent gene transcription. Furthermore, mutation (W199F) in the WW-domain of YAP1 abolishes its coactivation functions [21]. The coactivation function of YAP1 is strictly dependent on the PPXY motif of WBP-2. *The mode of action of WBP-2:YAP1 complex in ER and PR-mediated transactivation is not yet understood.* In this proposal, *we intend to dissect the molecular mechanism and significance of this interaction with relation to ER and PR function.*

As mentioned above, in addition to YAP1, we have also identified another WW-domain containing protein, WWOX1 (a tumor suppressor) as a WBP-2 interacting protein in our WW-domain array. WWOX1 was originally cloned as a putative tumor suppressor gene that spans one of the most common active fragile sites in the human genome, FRA16D. WWOX1 is located on chromosome 16q23.3 and exhibits genomic alterations in several cancer types and a recent study showed a possible involvement of methylation in the regulation of WWOX1 expression. WWOX1 expression level is high in normal tissues; its expression is highest in hormonally regulated normal tissues such as testis, prostate and ovary suggesting that WWOX1 may play an important role in hormone regulated cancers [32, 33, 66-71]. This suggestion is supported by the recent publications which show that WWOX1 expression is frequently altered in tumor tissues and introduction of WWOX1 into WWOX1-negative breast and prostate tumor cells resulted in tumor suppression and apoptosis both *in vitro* and *in vivo* [72]. The WWOX1 contains two WW-domains. The tandem WW-domains of WWOX1 play an important role in WWOX1 function. Both WW-domains of WWOX1 contain a central core of two consecutive aromatic amino acids and therefore belong to the class I specificity of domains, which recognize ligands with the PPXY consensus motif. Recent mapping of the WW-domain in the human proteome identified a

repertoire of PPXY-containing ligands that bind to individual domains of WWOX1. The first WW-domain of WWOX1 bound 18 and the second WW-domain bound 16 ligands, all with PPXY consensus. The mapping data clearly documented that although the second WW-domain of WWOX1 contains a tyrosine in the place of the second conserved tryptophan, the signature residue directly involved in ligand binding, the specificity of the "WY domain" toward PPXY core motif was not changed [33]. Previously, it has been shown that WWOX1 interacts with PPXY motif containing proteins, p73 and AP-2 and suppresses their transcriptional activities [36, 66, 73, 74]. Most recently, it has been shown that WWOX1 interacts with another PPXY motif containing protein, ErB-4 and compete with YAP1 for ErB-4 binding and suppress the coactivation functions of YAP1 [36]. Since, WWOX1 and YAP1 have the similar tandem WW-domains and both interact with common protein, WBP-2 but have different effects on transactivation, in this proposal, *we will examine whether WWOX1 and YAP1 expression have opposite effects on ER and PR-dependent gene transcription.*

PPXY Motif and WW-Domain; Possible Function in Gene Regulation: Protein-protein interaction modules are important for the proper signal transduction process in any cell. There are numerous such modules that mediate various processes from cell cycle progression to arrest and eventually apoptosis. One such module, the PPXY motif and WW-domain (PPXY-WW module) has gained prominence in the last decade. WW-domains mediate their interactions with proteins that contain a short PPXY motif. PPXY motif containing proteins essentially act as ligands for WW-domain containing proteins. The K_d of interaction for PPXY motif and WW-domain complex formation is in the high nM to low ìM values. Phosphorylation of the terminal tyrosine in the PPXY motif and phosphorylation of WW-domains containing proteins by specific kinases abolishes their interaction, suggesting that this modification represent a negative regulation mechanism for PPXY-WW module. Although the precise physiological roles of the PPXY motif and WW-domain remain undetermined, their presence in diverse proteins involved in signaling, regulatory, transcription and cytoskeletal functions, as well as their rapidly emerging role in signaling mechanisms that underlie several human diseases, clearly underscores their importance. Protein-protein interactions involving WW-domains and PPXY motif have been implicated in many diseases, including muscular dystrophy, Liddle's syndrome, Alzheimer's, Huntington disease and Cancers [16, 31, 35, 75].

Many new roles of the PPXY motif and WW-domain in the nucleus and transcription are just emerging. It is interesting to note that the PPXY motif is found in the transcriptional activation domains of many transcription factors and mutations in the PPXY motif either reduce or abolish their transcriptional activities. This observation suggests that the PPXY motif plays a role in mediating transcription stimulation by interacting with WW-domain containing proteins which act as transcriptional coactivator. Since, the interaction between WW-domain and PPXY motif is highly specific, it suggests that PPXY-WW complex is a more specialized coactivator complex of a subset of transcription factors [16, 21, 31, 35, 75]. This suggestion was supported by our observation which shows that PPXY motif containing protein, WBP-2 and WW-domain containing protein, YAP1 specifically coactivate ER and PR-dependent transcription where as this protein complex has no significant effect on the transactivation functions of other receptors. In contrast to this complex, it has been shown that other transcriptional coactivator complexes such as CBP, SRC-1 and p/CAF (p300/CBP associated factor) coactivates the transcriptional activities of a variety of receptors without exerting any specificity [6]. In addition to transcription stimula-

tion, the PPXY motif has also been shown to suppress transcription after interacting with certain WW-domain containing proteins. These observations suggest that *PPXY-WW module is involved in gene transcription but the actual mechanism by which PPXY-WW complex modulates transcription remains unknown.*

Significance: Coactivators play important roles in diverse pathological processes, such as cancer, inherited genetic diseases, metabolic disorders, and inflammation [6]. There is little doubt that we have much to learn about the biologically diverse roles of coactivators and that we have only scratched the surface of this expansive coactivator cosmos. Therefore, characterizing the mechanism of action of coactivator proteins will provide the prime source for the discovery of new molecular events in transcriptional reactions *and their role in cellular, physiological and pathological processes.* Collectively, our preliminary data suggests that WBP-2 (PPXY motif), YAP1 (WW-domain) and WWOX1 (WW-domain) are key regulators of ER and PR transactivation function but the precise roles of the PPXY motif and WW-domain containing proteins in steroid hormone receptor signaling [12], cell growth and carcinogenesis remain undetermined. Protein-protein interactions involving WW-domains and PPXY motif have been implicated in many diseases, including hormone regulated cancers. But the exact mechanism by which they regulate transcription, cell function and growth are largely unknown. Novel concepts and approaches to elucidate the molecular mechanisms by which PPXY motif and WW-domain containing proteins regulates hormone-dependent gene transcription are proposed here. Thus, accomplishing the specific aims outlined in this proposal will address the novel roles of the PPXY-WW complex in ER and PR function and will provide new and timely insights into the mechanism of action of PPXY motif and WW-domains in ER and PR signaling and cellular pathways that are regulated by these modulatory proteins.

Body

In the original proposal, we proposed to dissect the role od WBP-2 in ER and PR signaling and breast tumorigenesis.

A. Role of PY motif containing protein, WBP-2 in ER and PR signaling

B. Role of WW-domain containing proteins, YAP and WWOX1 in SHR function

C. Expression analysis of endogenous WBP-2 protein

A. Role of PY motif containing protein, WBP-2 in ER and PR signaling

This aim has been sucessfuly completed and the findings were reported in the previous annual report. The major out comes of this aim was also published

B. Role of WW-domain containing proteins, YAP and WWOX1 in SHR function

The third specific aim is intricately related to the second aim so we pursuing both the aims simultaneously. As a consequence of the modification of aim two the scope of this aim is extended to include the newly indentified WW-domain containing proteins that may be involved in the mechanism of action of WBP-2 protein.

I. Identification of WBP-2 Binding Proteins:

Specific protein-protein interactions and multiprotein complexes are important for a multitude of cellular processes including gene transcription. As mentioned above WBP-2 via its PPXY motifs binds to proteins that contain WW-domain. WW-domains are found in both cytoplasmic and nuclear proteins, WW-domains containing proteins are involved in ubiquitination, nuclear signaling, cell cycle control, transcriptional regulation and the recruitment of signaling proteins. Since, WW-domains bind to the PPXY motif and WBP-2 has been shown to contain PPXY motifs, an important step toward characterizing coactivation function of WBP-2 is to identify to which particular WW-domain containing protein it bind, and hence determine the mechanism by which it act as a coactivator. In order to identify the possible WW-domain containing proteins that could interact with the WBP-2 protein, we utilized Panomics' TranSignal WW-domain Array (Panomics Inc., CA, USA). This array contains 67 different human WW-domains from 42 different proteins. The arrays are made using the recombinant conserved binding sites of individual WW-domains fused with GST. Proteins are affinity purified and immobilized onto a membrane. Each WW-domain is spotted in duplicate. In order to identify the WW-domain(s) that interact with WBP-2, the full-length wild-type WBP-2 protein containing flag tag was expressed in bacteria. Afterward, WBP-2 protein was purified on flag beads and incubated with TranSignal WW-domain Array membranes. The protein-protein interaction was visualized by using HRP-based chemiluminescence detection. The resulting interacting proteins have been tabulated in table-1. Our screening data suggest that WBP-2 interacts with a wide variety of WW-domain containing proteins including YAP1, a transcriptional coactivator and WWOX1, a tumor suppressor (Fig. 1).

Table 1   List of WBP-2 interacting proteins

Gene Symbol	WW-domain(s)	Interaction	Protein name	Function
SMURF1	D1	-		E3 ubiquitin ligase
	D2	+		
SMURF2	D1	-		E3 ubiquitin ligase
	D2	+		
WWP1	D1	++	WW-domain containing protein 1	Nedd-4-like ubiquitin protein ligase
	D2	++		
	D3	++		
	D4	++		
NEDD4	D1	++	Neuronal precursor	Ubiquitin protein

	D2	++	cell-expressed devel-	ligase
	D3	++	opmentally down-	
	D4	++	regulated 4	
Caveolin-3		+		Membrane pro-tein
PABPN1		+	Poly(A)-binding pro-tein	
GAS7		+	Growth-arrest specific 7 isoform b	
YAP		++	*yes*-associated pro-tein	Transcriptional Coactivator
BAIAP1	D1	-	Membrane associ-ated guanylaste ki-nase-1	
	D2	+		
ITCH	D1	+	Atropin-1 interacting protein 4	E3 ubiquitin li-gase
	D2	+		
	D3	+		
	D4	+		
TAZ		++	Transcriptional co-activator with PDZ domain	Transcriptional coactivator
WWOX1	D1	++	WW-domain contain-ing oxidoreductase, isoform 1	Tumor suppres-sor
	D2	-/+		
APBB3		+	FE65-like protein	

II. PY3 motif of WBP-2 is essential for its interaction with YAP:

Initially, Sudol et al, identified WBP-2 as a binding partner for YAP using a functional screen. Our WW-domain array also confirms this observation that WBP-2 interacts with YAP. Next we wanted to know if this interaction is mediated by the PY motif of WBP-2 and the WW-domain of YAP, for this purpose we used both wild-type and coactivation function-dead PY3 motif mutant WBP-2 in GST pull-down assays. Figure 2, demonstrates that while the wild-type WBP-2 was able to interact with YAP, mutation of the PY3 motif of WBP-2 completely abrogated this interaction. Together with our previous observations we concluded that the PY3 motif of WBP-2, though not essential for its interaction with the receptors was indispensible for its interaction with the WW-domain containing protein YAP.

III. The WW-Domain Containing Protein, YAP1, Modulates Progesterone Receptor Transcriptional Activity via the WBP-2 Protein:

Since, WBP-2 binds to the WW-domain containing protein, YAP1 and YAP1 has been shown to be a transcriptional coactivator. Thus, we wanted to know whether YAP1 modulates steroid receptor-dependent target gene expression. To determine the role of YAP1 in steroid hormone receptor transactivation, HeLa cells were co-transfected with mammalian expression plasmids for the PR and ER receptors along with reporter plasmids containing their cognate hormone response element, with or without an expression vector for YAP1. *YAP1 alone did not affect PR-mediated transactivation either in the absence or presence of hormone.* In contrast, *when YAP1 was coexpressed with WBP-2 the hormone-dependent transcriptional activity of PR was significantly enhanced (~24-fold) (Fig. 3).* Similarly, YAP1 alone did not activate the ER-

mediated transactivation but when co-expressed with WBP-2 enhanced ER-mediated transactivation (data not shown). This activity was higher than the observed coactivation with WBP-2 alone (Fig. 3). ***These data suggest that YAP1 can modulate the ligand-dependent transcriptional activity of PR and ER but only via WBP-2.*** The PPXY motifs of WBP-2 have been shown to interact with the WW-domain of YAP1. Since our data revealed that the cPPXY of WBP-2 was required for its coactivation function, we next asked whether the cPPXY of WBP-2 is required for YAP1 to function as a coactivator for ER and PR. When coexpressed together, wild-type WBP-2 and wild-type YAP1 greatly enhanced the transactivation function of PR (Fig. 3). In contrast, the cPPXY mutant WBP-2 and wild-type YAP1 also failed to enhance the transcriptional activity of PR (Fig. 3). Our data demonstrate that *the cPPXY motif of the WBP-2 protein is required for YAP1 to function as a transcriptional secondary coactivator.* This data is consistent with previously published reports that YAP1 stimulates gene transcription by binding to the PPXY motif of ErbB4 protein. In order to determine whether the WW-domain of YAP1 is required for its transcriptional coactivator function, a WW-domain mutant (W199F) was utilized. This YAP1 mutant has been shown to be inactive in its ability to bind to PPXY motif. When coexpressed together, wild-type WBP-2 and WW-domain mutant YAP1, the WW-domain mutant YAP1 fail to act as a coactivator (data not shown). These data suggest that *WW-domain of YAP1 is required for it to function as a transcriptional coactivator.* In summary, our preliminary data *substantiate the role of WBP-2 (contains PPXY motif) and YAP1 (contains WW-domain) in female steroid hormone receptor function.* Based on our data, we postulate that *the cPPXY motif of WBP-2 binds to the WW-domain of YAP1 and recruits YAP1 to the target gene promoter by interacting with receptor. When the receptor-WBP-2-YAP1 complex is recruited to hormone responsive promoters, it acts at one of the many substeps required to modulate the transactivation functions of ER and PR- responsive target genes.*

IV. YAP Enhances Endogenous Estrogen Receptor Target Gene Activation via the WBP-2 Protein

To further substantiate that YAP and WBP-2 synergistically enhance ER and PR transactivation functions as observed by reporter gene assays, we performed real-time PCR (QPCR) analysis of ERα target gene pS2 in MCF-7 cells. MCF-7 cells were co-transfected with either control plasmid or expression plasmids of wild-type WBP-2, PY3 motif mutant WBP-2, and wild-type YAP alone or in combination. Cells were treated with either vehicle or estradiol (E$_2$) and expression of estrogen-regulated gene, pS2 was measured by QPCR. As control, we also examined the mRNA levels of ER, WBP-2 and YAP. There was no change observed in ER mRNA levels (data not shown) whereas, the mRNA levels of exogenously expressed WBP-2 (wild-type and PY3 mutant) and wild-type YAP were significantly increased in cells that were transfected with their expression plasmid compared to that of cells transfected with control plasmids (Figure 4A). As shown in Figure 4, WBP-2 enhanced the relative mRNA levels of ERα target gene, pS2 in the presence of estradiol but YAP alone showed no enhancement. Whereas, when YAP was coexpressed with wild-type WBP-2, the hormone-dependent transcriptional activity of ERα was synergistically enhanced, more than that observed with WBP-2 alone (Figure 4B Furthermore, this synergistic enhancement of pS2 relative mRNA levels was not observed when YAP was coexpressed with the PY3 motif mutant WBP-2. Taken together, both our reporter gene assays and endogenous target gene assays demonstrate that YAP can function as a secondary transcriptional coactivator (co-coactivator) of ERα in a WBP-2 dependent manner (Figure 4B).

V. Estrogen induces association of ERα with WBP-2 and YAP at ERα responsive gene pro-moter

Since WBP-2 and YAP interact with each other and synergistically enhance ERα transacti-vation functions, we next investigated the recruitment of WBP-2 and YAP to ERα responsive gene promoters by quantitative chromatin immunoprecipitation (ChIP) assays. YAP has been shown to be a potential transcriptional coactivator of various other transcription factors including p73 and RUNX2. We demonstrated previously the hormone dependent recruitment of WBP-2 to the ERα responsive pS2 promoter by classical ChIP assays in MCF-7 cells. To further investi-gate the association of ERα with WBP-2 and YAP at ERα responsive pS2 promoter we per-formed quatitative re-ChIP assays in MCF-7 cells. Cross-linked and sheared DNA-protein com-plexes from MCF-7 cells were immunoprecipitated with either non-specific purified IgG (Mock) or ER-α specific antibody. After immunoprecipitation the cross-linked immunocomplexes from each of the primary ChIPs were eluted and subjected to another round of immunoprecipitation with antibodies specific for WBP-2 or YAP. The immunocomplexes after re-ChIP were eluted, reverse cross-linked and the associated genomic DNA fragments were analyzed by QPCR with pS2 promoter specific primers.

We observed that in ER/WBP-2 ChIP assays where ERα antibody was used in the first ChIP and WBP-2 specific antibody was used in the re-ChIP, there was a significant enrichment in E_2-induced association of WBP-2 with ERα in comparison with the mock ChIP (IgG/WBP-2) as given by the increased association of pS2 promoter locus in QPCR assays (Figure 5). Similar results were observed with ER/YAP ChIP when compared to its mock (IgG/YAP) ChIP (Figure 5). These observations suggest that WBP-2 and YAP (as separate entities) are recruited to and are associated with ERα at the pS2 promoter locus in an estrogen dependent manner.

VI. Estrogen-induced recruitment of WBP-2 and YAP onto ERα responsive pS2 promoter is mu-tually interdependent

Our initial observations showed that YAP1 acts as a secondary coactivator of ERα only when co-expressed with WBP-2. We also show that estrogen enhances the recruitment of YAP1 to ERα responsive pS2 promoter. To test further if the recruitment of YAP1 is also de-pendent on WBP-2, we performed quantitative re-ChIP assays as described above in MCF-7 cells that were treated with siRNAs against YAP1 and WBP-2. As control in these assays, MCF-7 cells were treated with a non-specific scrambled siRNA.

In cells treated with non-specific scrambled siRNA, estrogen-induced association of ERα with WBP-2 (ERα/WBP-2) and YAP1 (ERα/YAP1) were consistent with our earlier observations when compared to their respective mock ChIP assays. Whereas in cells treated with siRNA against WBP-2, the E2-induced enhancement of association of YAP1 with ERα was completely lost, suggesting that the recruitment and association of YAP1 with ERα at the pS2 promoter lo-cus is dependent on the normal endogenous expression levels of WBP-2. Interestingly, the con-verse relationship also holds true, where the association of WBP-2 with ERα and its recruitment to the pS2 promoter locus was abolished in cells that were treated with siRNA against YAP1 (Figure 6). These observations suggest an intriguing possibility where the recruitment of WBP-2 and YAP1 to the pS2 promoter locus and their association with ERα may be mutually interde-pendent of their normal physiological expression levels in MCF-7 cells.

VII. WWOX1 suppresses the transcriptional coactivation functions of WBP-2 and YAP1

As mentioned above, we have also identified another WW-domain containing protein, WWOX1 as a WBP-2 interacting protein in our WW-domain array. WWOX1 interacts with WBP-2 via its first WW-domain. WWOX1 was originally cloned as a putative tumor suppressor gene and it has been suggested that WWOX1 may play an important role in hormone regulated cancers [32, 33, 70, 78, 79]. Previously, it has been shown that WWOX1 interacts with PPXY motif containing proteins, p73 and AP-2 and suppresses their transcriptional activities. Most recently, it has been shown that WWOX1 interacts with another PPXY motif containing protein, ErB-4 and compete with YAP1 for ErB-4 binding and suppress the coactivation functions of YAP1 [74]. Since, WWOX1 and YAP1 have the similar tandem WW-domains and both interact with common protein, WBP-2; we ask whether WWOX1 and YAP1 expression have opposite effects on ER and PR-dependent gene transcription. To determine the role of WWOX1 in steroid hormone receptor transactivation, MCF-7 cells were co-transfected with ER-responsive reporter plasmid along with expression vectors for either WBP-2, YAP1, and WWOX1, WBP-2 and WWOX1 or WBP-2, YAP1 and WWOX1. As shown before, WBP-2 coactivates the transactivation functions of ER. Furthermore, YAP1 and WWOX1 alone had no significant effect on ER function. But when YAP1 was coexpressed with WBP-2 the hormone-dependent transcriptional activity of ER was synergistically enhanced (Fig. 7). In contrast, expression of WWOX1 significantly reduced the coactivation functions of WBP-2. Similarly, WWOX1 also significantly suppressed WBP-2-YAP1-mediated transcriptional activities of ER and PR in a dose-dependent manner (Fig. 7). To determine whether the WW-domain of WWOX1 is required for its transcriptional suppression function, a WW-domain mutant was utilized in which the tryptophan 33 within WW-domain 1 was mutated to arginine. This WWOX1 mutant has been shown to be inactive in its ability to bind to PPXY motif. When coexpressed along with wild-type WBP-2 and wild-type YAP1, this mutant had no significant effect on WBP-2 and YAP1's coactivation functions (Data not shown). Identical results were obtained with PR (data not shown). These data indicate that *the coactivation functions of WBP-2 and YAP1 are suppressed by WWOX1, suggesting that WWOX1 may regulates the transactivation functions of ER and PR by antagonizing the functions of WBP-2 and YAP1.*

C. Expression analysis of endogenous WBP-2 protein

In light of the recent modifications to the original proposal we have accommodated and updated the first aim of the proposal. In the original proposal we intended to analyze only the expression profile of WBP-2 in various cancer cell lines and human breast tissue arrays. Our current understanding is that YAP1 and WWOX1 may play vital roles in the regulations and function of WBP-2, furthermore YAP1 has been shown to be amplified in various breast cancers and intriguingly, WWOX1 has been show to be a potent tumor suppressor [74]. Given these interesting facts we propose to analyze the expression of WBP-2, YAP1 and WWOX1 in correlation with the ER and PR expression status of various breast cncer cell lines as well as many breast tumor-arrays which may shed some light on the complicity of WBP-2 function and the possible roles of YAP1 and WWOX1 in breast cancers.

We did Immunohistochemistry (IHC) to explore the localization and expression level & pattern of specific proteins in cells of tumor& normal tissues of female breast. Tissue Micro Arrays (TMAs) 0f 4µm thick ness, paraffin embedded, formalin fixed Breast cancer –normal-adjacent (60 samples) & Breast cancer-metastasis-normal-adjacent (60 samples) were analyzed for expression of ER, PR, WBP-2, WWOX and YAP. TMAs were de paraffinezed in xylene

& rehydrated through graded alcohol. Antigens retrieved through Citrate based buffer soln and quenched the endogenous peroxidase activity with 3% H2O2   in methanol. Sections were blocked with 10% normal goat serum to block the non-specific immunoreactivity. Primary Antibodies for ER, PR, WBP-2, WWOX and YAP were applied at appropriate dilutions. Biotinylated secondary antibodies were applied for binding to specific primary antibodies. For visualization we used Avidin Biotin Complex technique with 3, 3 Diamnobenzidine (DAB) for chromogenic reaction. Hematoxylin was used as counter stain (Fig. 9).

Dr Mercy Jorda did pathology and quantification of expression profile of TMAs. Staining intensity for IHC, based on immunoreactivity of tumor cells that provide more assessment of predictive and prognostic value.

Degree of staining intensity varied by tumor type in most of the cases. Normal sections are used as control for tumor sections & level of expression of specific proteins showed the behavior of that specific protein while its localization tells the pattern of expression. Tumor samples were either ER & PR negative or showed high expression. Focal expression was high in normal sections. Most of the samples that were positive for WWOX were also positive for WBP-2. Both localized more in the cytoplasm. The effect of WWOX and YAPduring breast carcinogenesis may be through interaction with steroid receptor signaling pathways, a hypothesis that could be explored by using ER & PR negative or ER & PR positive cell lines. YAP localized in the nucleus of normal duct, dense and showed high expression. It expressed in the cytoplasm of tumor duct, diffused in most of the samples.

Table 2:   Summary of Immunohistochemistry analysis

Fisher Exact Test

	NORMAL		TUMOR		
Total(120)	40		80		P Value
	Neg (%)	Pos (%)	Neg (%)	Pos (%)	
ER	23(57.5)	17(42.5)	63(78.75)	17(21.25)	0.0188
PR	38 (95)	02 (05)	73(91.25)	07 (8.75)	0.7161
WBP-2	06 (15)	34 (85)	36 (45)	44 (55.0)	0.0011
WWOX	16 (40)	24 (60)	11(13.75)	69(86.25)	0.0022
YAP	06 (15)	34 (85)	18 (22.5)	62 (77.5)	0.4684

To check the significance of these protein we applied Fisher Exact Test. Pearson value for ER is significant while Pearson value for PR is non significant as is shown in **table 2** High expression of ER is an early event in breast carcinogenesis while decrease of PR level is associated with breast cancer progression. Pearson value for WBP-2 & WWOX is highly significant. While for YAP is non significant as appears in this table but if we look into its expression in cytoplasm it appears highly significant (data not shown) the reason may be its involvement in different pathways when localized differently. Small cohort size was a limitation. We will do more TMAs that include different stages of breast cancer to get significant statistical number for better assessment and will test cytokeratin5/6, HER-2 for the same and rest of the TMAs to study further the role of these proteins in Triple Negative Tumors.

The assessment of ER and PR status and its co regulators in tumors by immunohistochemistry analysis provides clinicians with important prognostic & diagnostic information, and helps predict the response to endocrine therapy.

Key Research Accomplishments

- Establishing that WBP-2 is a coactivator of ER and PR

- The PY motif of WBP-2 is essential for its coactivation function

- Identification of YAP1 and WWOX1 as WBP-2 interacting proteins

- YAP1 acts as transcriptional secondary coactivator of ER and PR that is strictly dependent of WBP-2

- WW-domain of YAP and the PY motif of WBP-2 are essential for their coactivation activities

- WBP-2 and YAP are recruited to ER-responsive promoter

- Estrogen dependent recruitment and association of WBP-2 with ER is dependent on YAP, and vise versa.

- WWOX1 may act as a transcriptional repressor of WBP-2

Reportable Outcomes

1. The first part of the this work has been published "Dhananjayan, S.C., et al., *WW Domain Binding Protein-2, an E6-Associated Protein Interacting Protein, Acts as a Coactivator of Estrogen and Progesterone Receptors.* Mol Endocrinol, 2006. **20**(10): p. 2343-2354" (Please refer to previous Annual Report).

2. The second part of this report was presented at the Annual Endocrine Society Meeting, ENDO 2007 (June 2-5th), in Toronto, Canada

3. The third section of this project is being written up as a manuscript to be submitted to Cancer Research.

Conclusions

Our data demonstrates that WBP-2 is recruited onto the hormone responsive promoters in the presence of hormone and it specifically enhances the transactivation functions of PR and ER. Our data also demonstrates that WBP-2 contains an intrinsic activation domain and the cPPXY of WBP-2 is essential for its coactivation and intrinsic activation functions. Our preliminary data also demonstrates that the WBP-2 binding protein, YAP1 enhances PR and ER transactivation but YAP1's coactivation function is absolutely dependent on WBP-2. Furthermore, cPPXY motif of WBP-2 and WW-domain of YAP1 is required for YAP1 to work as a transcriptional coactivator. Additionally, our data also indicate that the coactivation functions of WBP-2 and YAP1 are suppressed by WWOX1, suggesting that WWOX1 may regulates the transactivation functions of ER and PR by antagonizing the functions of WBP-2 and YAP1 (Fig. 8). Taken together our data established the role of WBP-2 and YAP1 as coactivators and WWOX1 as a repressor for ER and PR transactivation pathways.

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Appendix 1

Figures
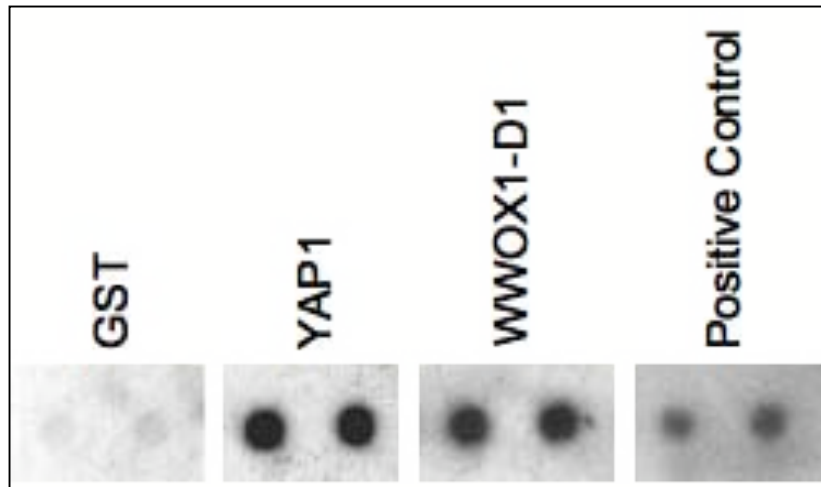
Fig. 1: WBP-2 interacting proteins
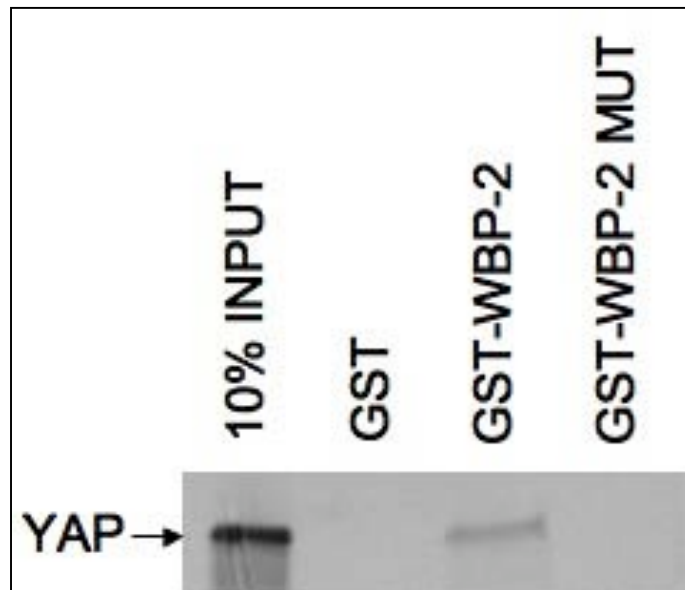
Fig. 2: PY motif of WBP-2 is essential for interaction with YAP1
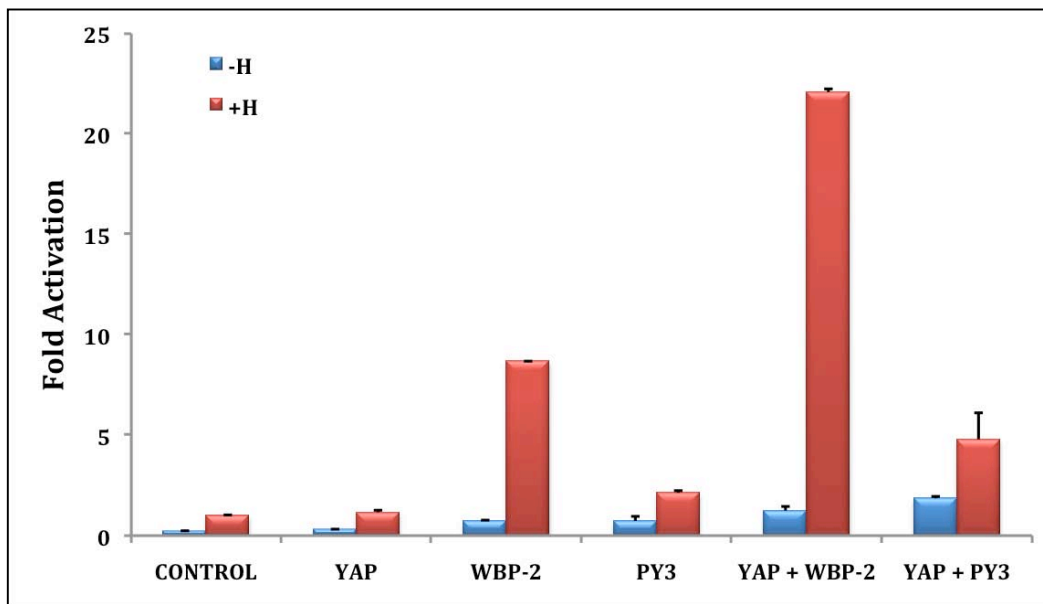
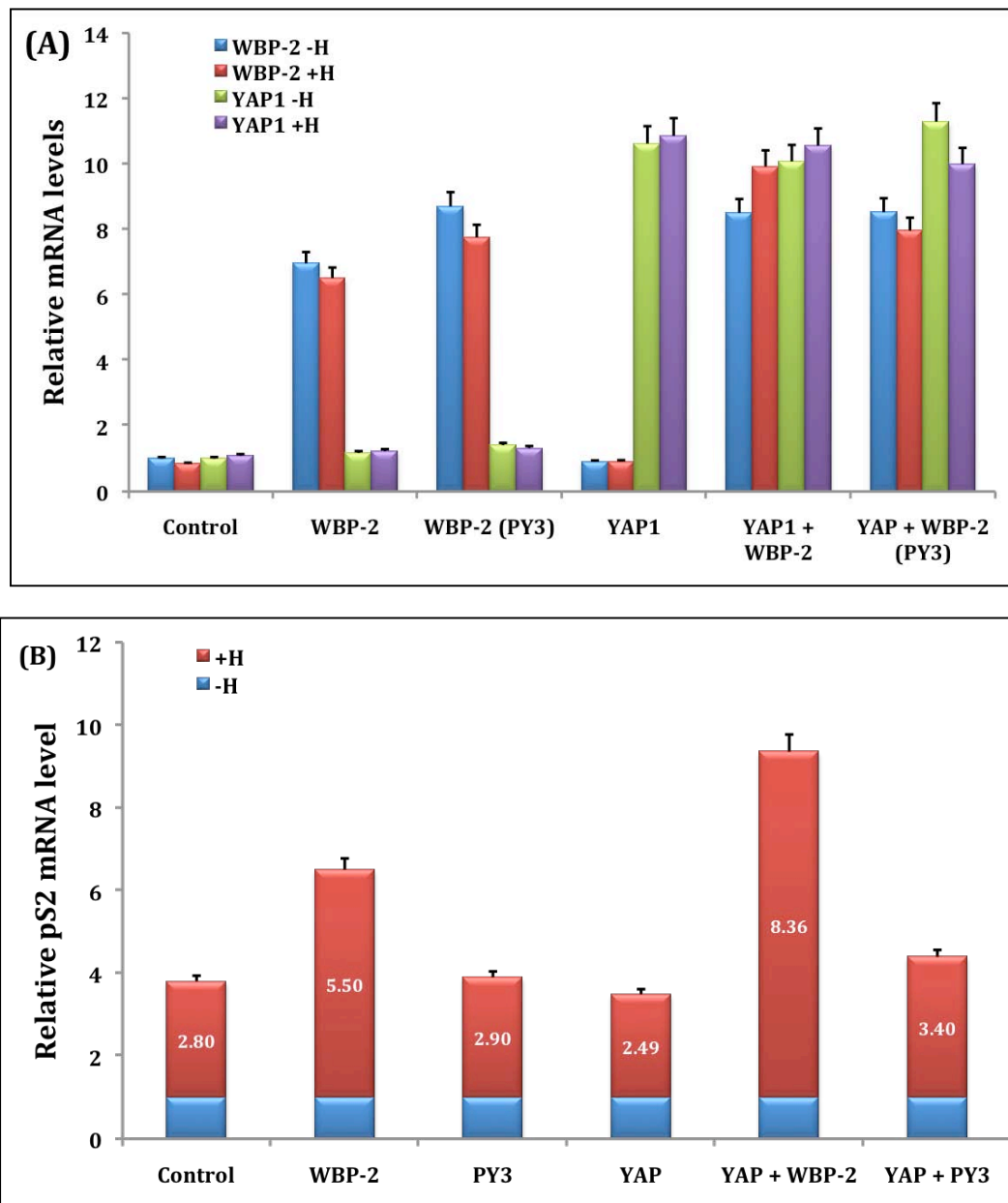Fig. 3: YAP coactivation function is dependent on the PY motif of WBP-2

Fig. 4: A. WBP-2 and YAP1 mRNA expression levels after transfection. B. YAP-mediated coactivation of endogenous ER target gene, pS2.
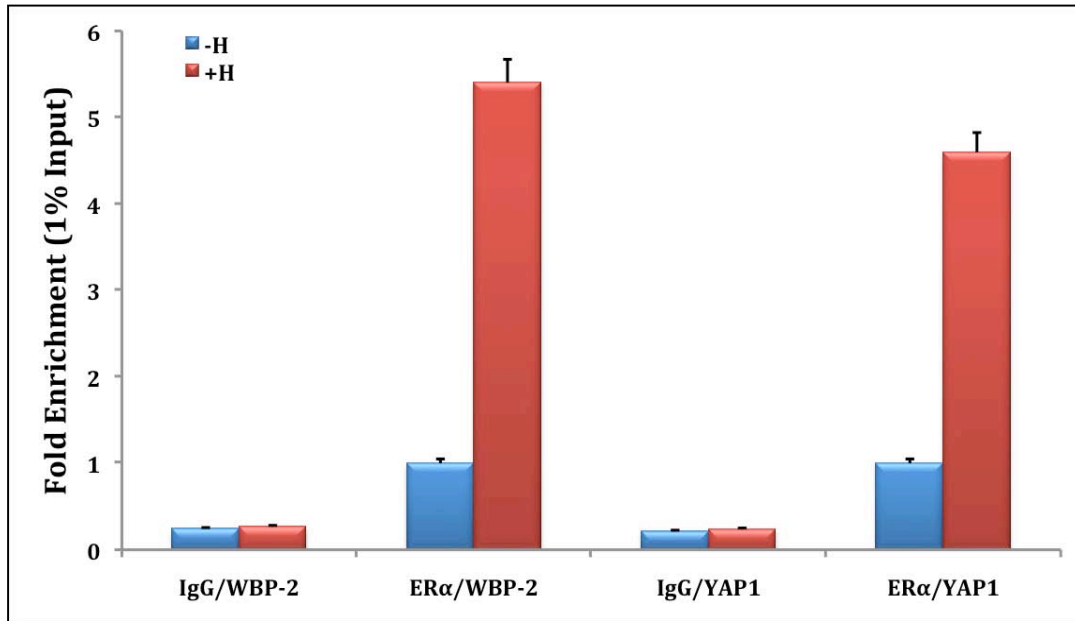
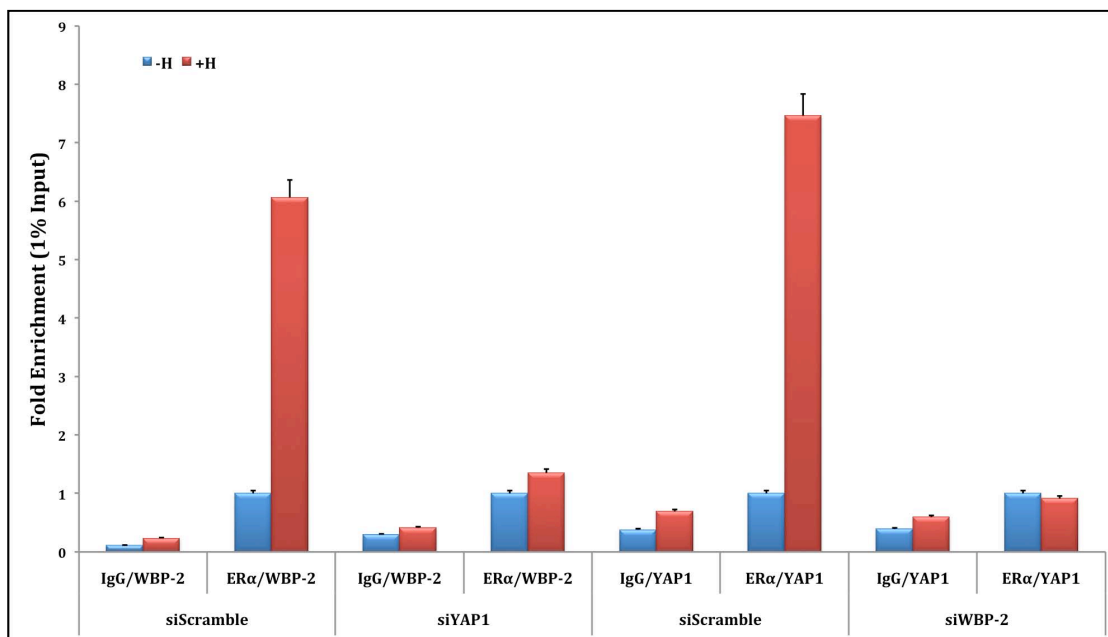Fig. 5: WBP-2 and YAP are associated with ERα at the pS2 promoter in an estrogen dependent manner.

Fig. 6: Estrogen dependent recruitment and association of WBP-2 with ERα is dependent on YAP, and vise versa.
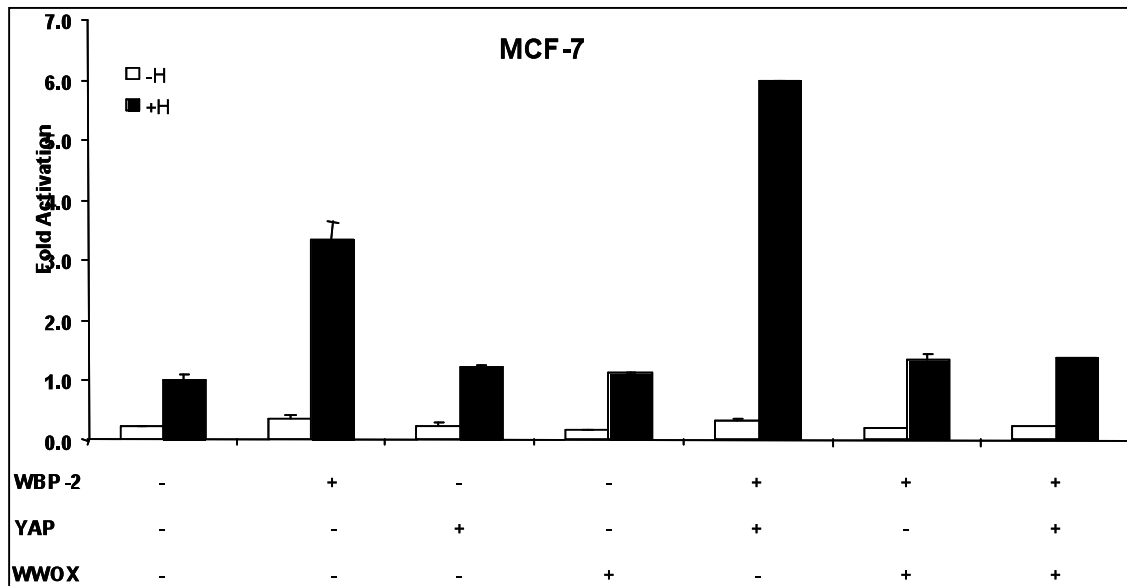
Fig. 7: WWOX1 represses the coactivation
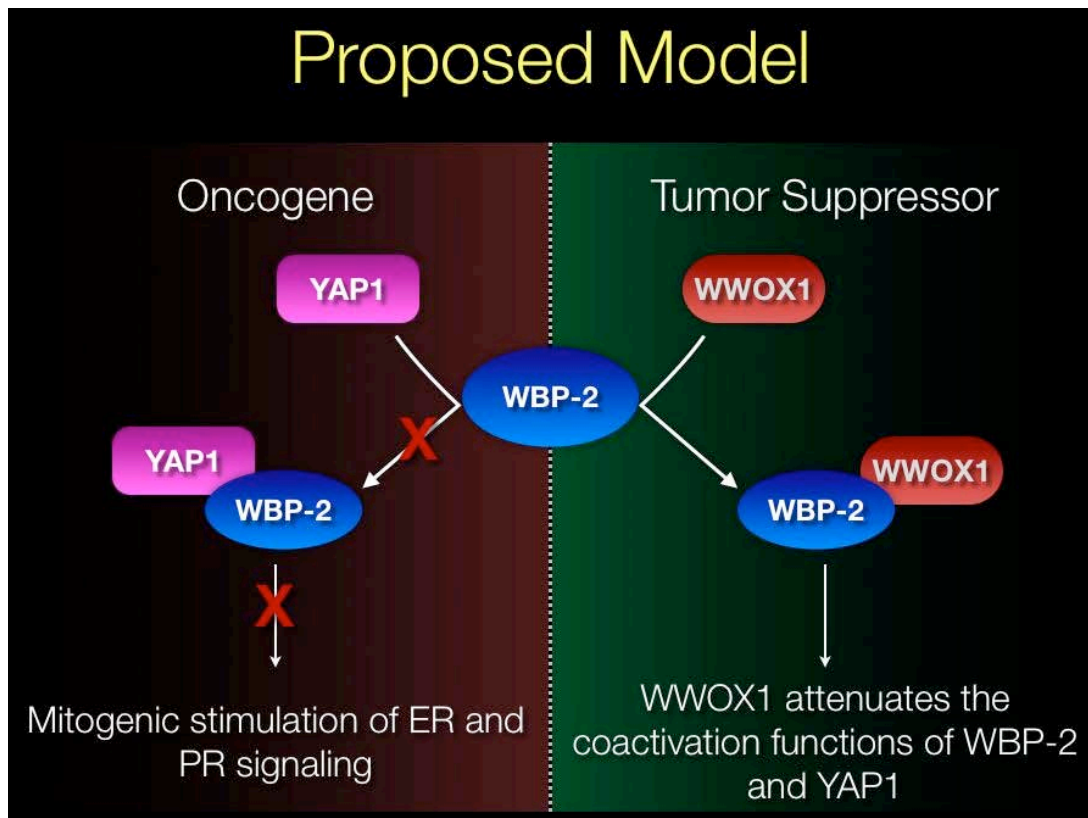functions of WBP-2 and YAP1

Fig. 8: Proposed model for the role of WW-domain containing proteins, YAP and WWOX1 in WBP-2-mediated ER and PR signaling
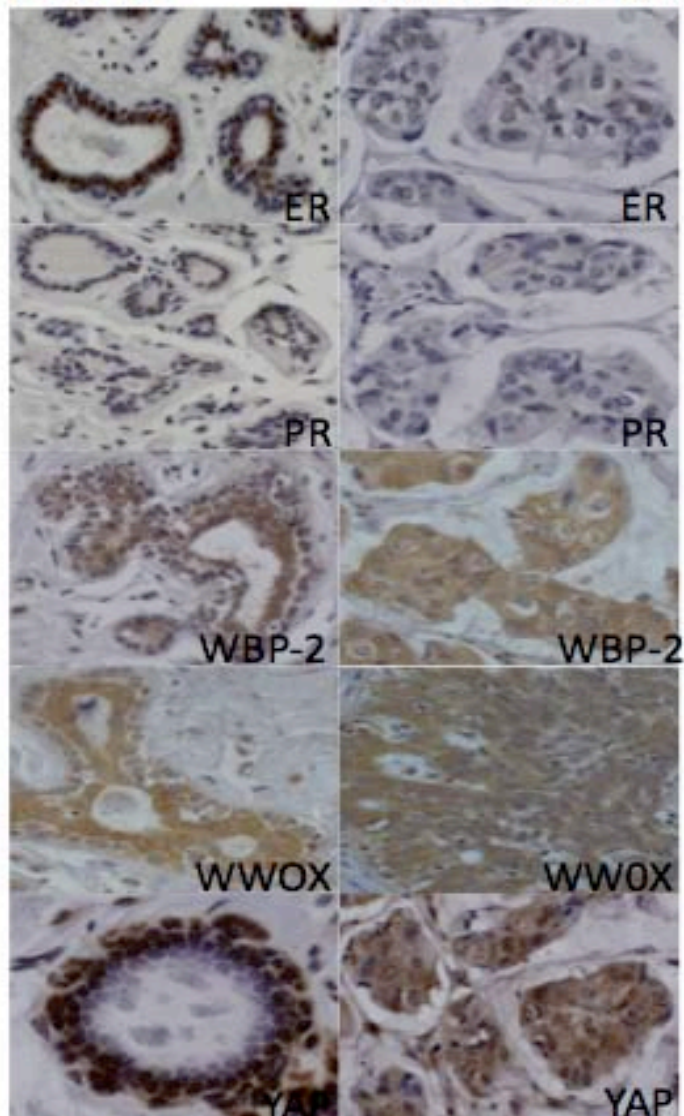
Fig. 9: Immunohistochemistry analysis for ER, PR, WBP-2, YAP1 and WWOX1 in various breast cancer patient sample tissue microarrays.